

A Plaque Assay for Chick-Embryo-Lethal-Orphan (CELO) Virus¹ (35297)

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In recent years, this laboratory has been interested in the oncogenic potential of various adenoviruses (1). During studies with CELO virus, an oncogenic avian adenovirus (2, 3), which also transforms cells *in vitro* (4, 5), we attempted to develop a quantitative plaque assay for the virus. Although a plaque assay has been described (6, 7), in our experience, the host cells proved difficult to maintain under an agar overlay for a sufficient time to allow plaques to develop and be counted. Using the protamine sulfate method described by Tytell *et al.* (8), a readily reproducible plaque assay for CELO virus in chick kidney cells was found.

Materials and Methods. Chick kidney cells (CKC) were prepared from 21-day-old chick embryos. The kidneys were removed and washed with Hanks' balanced salt solution (HBSS) containing 200 $\mu\text{g}/\text{ml}$ of kanamycin sulfate (Kantrex, Bristol Laboratories). After washing the tissues free of erythrocytes, 125 ml of 0.25% trypsin was added to a flask containing 36 pairs of kidneys. The tissue was trypsinized for 1 hr at 37°, using a magnetic stirrer. The cell suspension was filtered through 2 layers of sterile gauze and centrifuged for 10 min at 1500 rpm. The cells were resuspended at a ratio of 300 ml of growth medium/ml of packed cells. Cells were grown in Eagle's minimum essential medium (MEM) containing HBSS and supplemented with 10% heat-inactivated fetal calf serum (FCS). Erythromycin (50 $\mu\text{g}/\text{ml}$) and streptomycin (100 $\mu\text{g}/\text{ml}$) were added to the growth medium. Five ml of the cell suspension were inoculated into 60-mm plastic petri plates (Falcon) and incubated

for 2–3 days at 37° in a mixture of 5% CO₂ in air.

Monolayers were washed 3 times with 5 ml of HBSS containing 2% agamma calf serum (AgCS) and kanamycin (200 $\mu\text{g}/\text{ml}$). Virus dilutions were prepared in HBSS with 2% AgCS and 1 ml of the virus suspension was inoculated/plate. After adsorption for 3 hr at 37°, 5 ml of overlay medium were added to each plate.

The overlay media used are listed in Fig. 1. The liquid components were prepared as double strength stocks and were diluted with an equal volume of 2% Bacto agar (Difco) prior to use. All media contained a final concentration of 50 $\mu\text{g}/\text{ml}$ of erythromycin, and 100 $\mu\text{g}/\text{ml}$ of streptomycin. A 1% protamine sulfate (Salmine, Nutritional Biochemicals) stock solution was prepared in triple distilled water, autoclaved at 15 lb pressure for 30 min, and stored at 4° in 50-ml aliquots. This stock was diluted 1:25 in the final overlay medium (11). In some experiments, kanamycin sulfate, 200 $\mu\text{g}/\text{ml}$, and nystatin (Mycostatin, Squibb and Sons, Inc.), 50 units/ml, were added to the overlay. Five ml of overlay medium were delivered/plate. After 5–6 days of incubation at 37°, an additional 5 ml overlay was added. On the day before counting, 3 ml of 1% agar containing 0.04% neutral red were added to each plate and plaques were counted after overnight incubation at 37°.

The virus used was the Phelps strain of CELO virus (9), plaque purified 3 times in CELO-free chick kidney cells (Spafas Inc., Norwich, Conn.). Stocks were prepared by inoculating 10⁶ plaque-forming units (PFU)/0.1 ml into the allantoic cavity of 10-day-old embryonated CELO-free eggs, and harvesting the fluids 72 hr later. The titers of the

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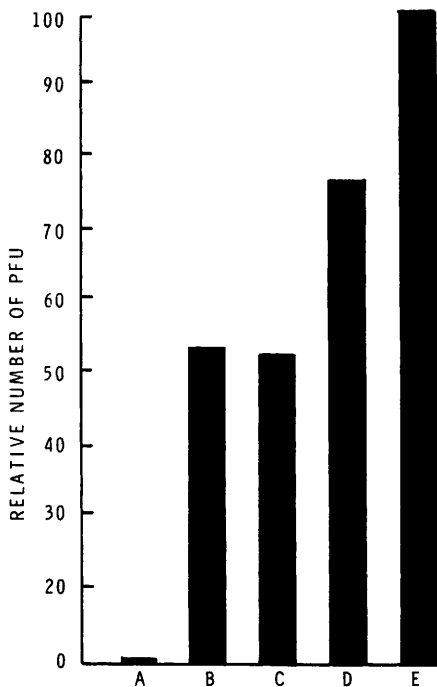


FIG. 1. Effect of media on plaque production by CELO virus: (A) MEM, 2.5% CS, 0.22% NaHCO_3 , 0.04% protamine SO_4 + 1% agar; (B) MEM, 2.5% FCS, 0.035% NaHCO_3 , 1% agar; (C) MEM, 2.5% FCS, 0.035% NaHCO_3 , 0.04% protamine SO_4 , 1% agar; (D) MEM, 2.5% FCS, 0.22% NaHCO_3 , 1% agar; and (E) MEM, 2.5% FCS, 0.22% NaHCO_3 + 0.04% protamine SO_4 , 1% agar. Cells could not be maintained under an agar overlay containing Medium A.

virus stocks were approximately 1×10^9 PFU/ml.

Results. Media. To test the effect of various overlay media on plaque production by CELO virus, monolayers of CKC were infected with 125 PFU of virus and allowed to adsorb for 3 hr at 37° . Groups of 6 plates were overlaid with medium in which the type of serum, concentration of bicarbonate, and protamine sulfate were varied. A second overlay was added on the 6th day. After addition of neutral red, the plaques were counted on the 11th day. The formulations for the media and the results obtained are shown in Fig. 1. The greatest plating efficiency and the largest plaque size were obtained on plates overlaid with media containing 2.5% FCS, 0.22% bicarbonate, and 0.04% protamine sul-

fate. Deletion of protamine sulfate from this medium decreased the total number of plaques; however, plaque size remained the same. When the bicarbonate concentration was lowered to 0.035%, with or without the addition of protamine, the plaque-forming ability of the virus was decreased. Plaques formed on these plates were pinpoint in size and difficult to count. A comparison of plaque size in these cultures to those containing 0.22% bicarbonate is illustrated in Fig. 2. Replacing the 2.5% FCS with 2.5% calf serum (CS) resulted in degeneration of the cell sheet by the 11th day. The medium containing 2.5% FCS, 0.22% bicarbonate and 0.04% protamine sulfate, was used in all subsequent experiments.

Enumeration. Groups of 4 plates were infected with 125 PFU of CELO virus. To determine optimum time for plaque counting groups of plates were overlaid with neutral red on the fifth day, and every other day thereafter, and the plaques counted after overnight incubation at 37° . Results are summarized in Table I. Counts were low on day 6, but reached a plateau from days 8 through 12. Plaque size increased from days 6 through 14. On days 6 and 8, plaque size was too small for reliable counting. By day 14 plaques were so large as to be confluent. Day 10 was selected as the optimum time for counting.

Additional experiments established a linear relationship between the number of plaques counted and virus dilution.

Time of adsorption. The optimal adsorption time necessary for maximum plaque production was also determined. A virus dilution containing 100–125 PFU was allowed to ad-

TABLE I. Number of Plaques Related to Day of Counting.^a

| After infection (day) | Mean no. of plaques \pm SD |
|-----------------------|------------------------------|
| 6 | 58 \pm 16.2 ^b |
| 8 | 121.3 \pm 32.5 |
| 10 | 122.8 \pm 15.3 |
| 12 | 123 \pm 3.0 |

^a Neutral red added approximately 20–24 hr before counting plaques.

^b Pinpoint plaques.

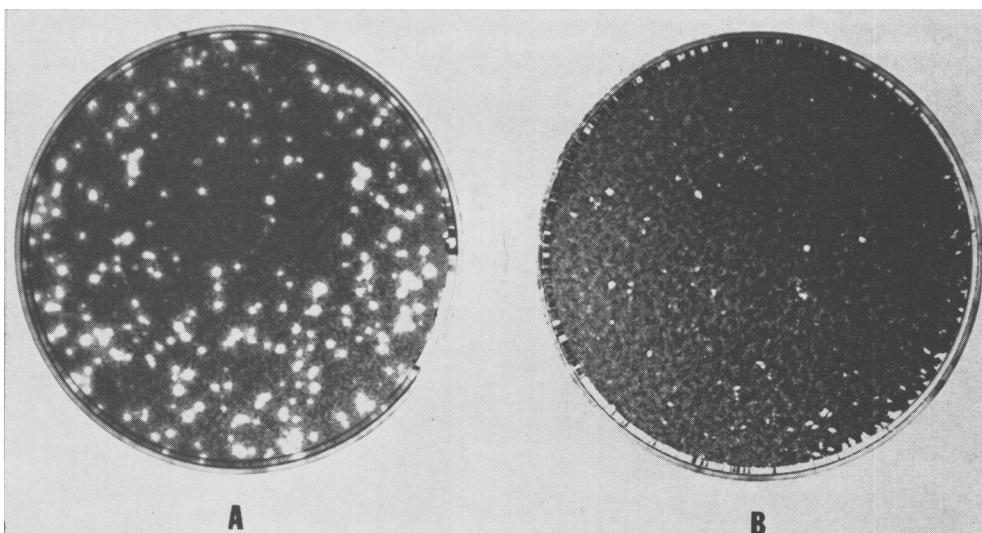


FIG. 2. CELO virus plaques in chick kidney cells stained with neutral red: (A) overlaid with medium containing 0.22% bicarbonate; (B) with medium containing 0.035% bicarbonate. Note increase in plaque size with 0.22% bicarbonate (actual size).

sorb to the cells at 37° using four plates for each time interval. After adsorption, the inoculum was decanted and the plates were washed once with HBSS containing 2% AgCS before overlaying. The results are summarized in Fig. 3. Maximum plaque counts

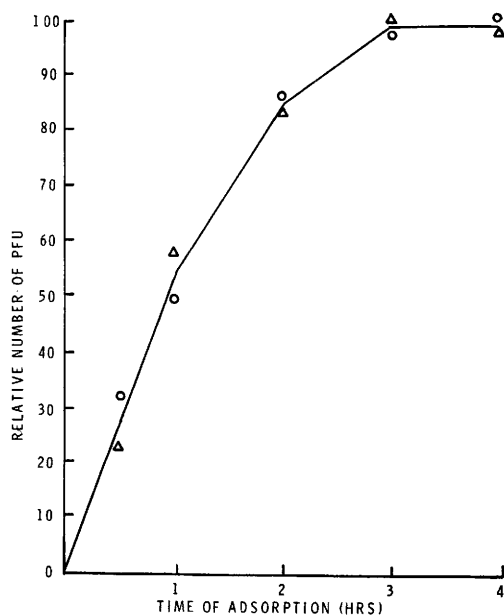


FIG. 3. Adsorption of CELO virus to chick kidney cells. Each point is the mean of 4 replicate plates.

were obtained after a 3-hr adsorption period. A longer adsorption period did not significantly alter the number of plaques observed.

Discussion. Protamine sulfate may be utilized by cells to maintain a viable state for extended periods under agar (8). The protamine may serve as a source of arginine for both the replication of the virus and maintenance of cell monolayers (11). The necessity of arginine for adenovirus replication has been shown by Rouse *et al.* (10). In our experiments, the addition of protamine sulfate produced higher plaque counts with CELO virus and the medium has also proved useful in plaquing simian adenovirus type 7 in BSC-1 cells, and infectious canine hepatitis virus, a canine adenovirus, in MDCK cells.

Plaque size and plaque number decreased when the bicarbonate concentration was lowered to 0.035%. Media containing increased amounts of bicarbonate increased the plaque size even in the absence of protamine sulfate. Therefore, plaque size appeared to be a function of the bicarbonate concentration; the number of plaques, a function of the protamine.

Since chick kidney cells are relatively difficult to maintain using an agar overlay, this medium may prove useful in plaquing other

avian viruses in chick kidney cells.

Summary. A procedure was described for plaquing of avian adenovirus (CELO) in chick kidney cells. The media consisted of Eagle's MEM with 2.5% fetal calf serum, 0.22% sodium bicarbonate and 0.04% protamine sulfate, in 1% Bacto agar. Using this medium, monolayers of chick kidney cells could easily be maintained for at least 12 days. Plaques could be counted on the 11th day, after addition of neutral red (0.04%) on the 10th day. An adsorption period of 3 hr at 37° gave optimal plaque counts.

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